



De novo Assembly, Characterization and Functional Annotation of Southern Hake (*Merluccius australis*) Transcriptome

Daniela Reyes¹, John Gold², Ruth González¹ and Rodrigo Vidal^{1*}

¹ Laboratory of Molecular Ecology, Genomics and Evolutionary Studies, Department of Biology, Universidad de Santiago, Santiago, Chile, ² Marine Genomics Laboratory, Department of Life Sciences, Center for Biosystematics and Biodiversity, Harte Research Institute, Texas A and M University, Corpus Christi, TX, USA

OPEN ACCESS

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*Correspondence:

Rodrigo Vidal
ruben.vidal@usach.cl

Specialty section:

This article was submitted to
Marine Fisheries, Aquaculture and
Living Resources,
a section of the journal
Frontiers in Marine Science

Received: 13 August 2016

Accepted: 20 October 2016

Published: 10 November 2016

Citation:

Reyes D, Gold J, González R and
Vidal R (2016) De novo Assembly,
Characterization and Functional
Annotation of Southern Hake
(*Merluccius australis*) Transcriptome.
Front. Mar. Sci. 3:216.
doi: 10.3389/fmars.2016.00216

Southern hake (*Merluccius australis*) is an ecological and economically important demersal fish in Chile and Argentina. Notwithstanding, genetic resource for genetic or ecological studies on this species are scarce. Consequently, here we present transcriptome sequencing results (RNA-Seq) for spleen and liver tissues with the 454 FLX titanium platform. The *de novo* transcriptome assembly generated 10,314 unigenes, with an average length of 510 bp, N50 of 572 bp and 3171 annotated sequences. A specific Gadiform BLAST search, with focus on immune genes, showed 186 (56%) genes homologous to those of Atlantic cod. A total of 2302 microsatellites were detected in 1687 unigenes and 741 presented adequate flanking sequences for primer design. In total, these potential molecular markers and transcriptome characterization represent an important resource for genetic and ecological studies on southern hake.

Keywords: microsatellite, southern hake, RNA-Seq, transcriptome, spleen, kidney

INTRODUCTION

The *Merluccius* genus is a widely distributed and important group in marine fisheries, which resides in the continental shelf and slope (Quinteiro et al., 2000). Although many taxonomic studies addressing this genus have been published, the most comprehensive ones conclude that this genus contains about 12–13 species and several subspecies (Inada, 1981; Cohen et al., 1990). The southern hake, *Merluccius australis*, is a circumglobal demersal fish with two described populations or subspecies described (Lloris et al., 2005). One subspecies denominated *M. australis polylepis* is found primarily from 40°S in the southeast Pacific (Chile) southwards around the southern tip of South America, to 38°S in the southwest Atlantic (Argentina), and another subspecies called *M. australis* is present in Chatham Rise, Campbell Plateau and South Island northward to the East Cape. This species comprises one the most economically important industrial and artisanal fisheries in the Southern Hemisphere (Alheit and Pitcher, 1995). It also plays a critical ecological role in the marine ecosystem (Ojeda et al., 2011). Moreover, the southern hake is considered a potential farming species and its status corresponds to the phase of demonstration, where its commercial potential is being evaluated (Bricknell et al., 2006). Despite its nutritional, ecological and economic importance, genomics resources and research on this species are scarce. This is illustrated, for example, by the low number of specific molecular markers and genome sequences available for this species (Machado-Schiaffino and Garcia-Vazquez, 2009; Mabragaña et al., 2011; Renshaw et al., 2011). In June 2016, there was no information on southern hake EST available in NCBI dbEST.

This shows clear contrast with another relevant hake species, for example, the European hake (*Merluccius merluccius*), for which a number of transcriptomic and molecular markers resources have been recently developed (Milano et al., 2011, 2014).

In last years, transcriptome analysis was recognized as a key tool for the development and improvement of several aquaculture species (McAndrew and Napier, 2011). Availability of resources in the genome level for southern hake will enhance its genetic and ecologic studies, aquaculture development and contribute to future comparative genomic research on the *Merluccius* genus. Therefore, in this study we conducted 454 pyrosequencing of one cDNA library isolated from southern hake. Our goals were to (i) characterize the kidney and liver transcriptome of southern hake (*M. australis polylepis*) and (ii) describe and characterize molecular markers for future genetic analyses. We choose kidney and liver tissues, because both have been described as key organs for immune defense in fishes (Lieschke and Trede, 2009) and its transcriptome characterization will have important utility for both scientific and applied purpose.

MATERIALS AND METHODS

Tissue Collection and RNA Extraction

Spleen and liver tissue were collected by fisherman from 10 commercial caught wild adult individuals (41°28' S-72°56' W). All the animal experiments in this study were approved and conformed to the Institutional Ethics Committee, Universidad de Santiago, guidelines. Initially, the tissue were placed in RNAlater buffer (Ambion Inc.) and stored at 4°C by 24 h and then stored at -20°C. Total RNA was extracted according to Cepeda et al. (2011). The concentration and integrity of the RNA were evaluated by spectrophotometry (Nanodrop) and by Agilent Bioanalyser 2100, respectively.

cDNA Library and Sequencing

The MINT-2 kit (Evrogen) was utilized for cDNA synthesis according to the manufacturer's instructions. Double-stranded cDNAs were submitted to McGill University and Genome Québec Innovation Centre for library construction and sequencing. One cDNA library was constructed from pooled equal amounts of RNA from both tissues and sequencing was conducted on a 454 GS FLX titanium instrument.

Sequence Pre-processing and *De novo* Assembly

The pre-processing of the obtained reads was performed using CLC Genomics Workbench tools version 7.0.3 (CLCBio) and included the trimming of polyA tails, adaptors and low quality fragments (quality score limit = 0.05, ambiguous nucleotides = 2), as well the elimination of duplicated reads and reads with length below 50 base pairs (bp). Processed reads were *de novo* assembled with CLC *de novo* assembly tool (minimum contig length = 100, deletion cost = 3, insertion cost = 3, mismatch cost = 2, length fraction = 0.5, similarity fraction = 0.5) and MIRA3 (job = *de novo*, est, accurate, 454, 454_SETTINGS -AL:mo = 40, -SK:pr = 97) (Chevreux et al., 2004) programs.

The obtained contigs from both programs were merged and re-assembled with the CAP3 program (default parameters, except to overlap = 40, identity = 97) (Huang and Madan, 1999) to improve the credibility of the assembly (Kumar and Blaxter, 2010).

Sequence Annotation

The functional annotation of the obtained contigs was done through a local alignment against Swiss-Prot database (Bairoch and Apweiler, 2000) and NCBI non-redundant protein database (nr) (June 2016) using the BLASTX program (Camacho et al., 2009), with E-value cut off equal to 1e-3 and 1e-6, respectively. Gene Ontology (GO) terms and enzyme commission (EC) associated to the blast hits were recovered using the BLAST2GO suite tools (Götz et al., 2008), whereas metabolic pathways assignments were performed using tools supplied by Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000).

Southern Hake Transcriptome Completeness

Parra et al. (2007) have developed a list of 248 highly conserved core eukaryotic genes (CEGs), which can be considered as an appropriate indicator of completeness of gene cover in eukaryotic organisms. We use BLASTX to map the transcripts obtained of southern hake to the CEGs; hit of $e \leq 1.0e-12$ were considered as a match. To further characterize the southern hake transcripts related with immune function, the term corresponding to "immune system" GO level 2 (and its respective children) of Atlantic cod (*Gadus morhua*; gadMor1) were downloaded from ENSEMBL (BioMart). The ENSEMBL codes were used to download the corresponding protein sequences. Then, we used BLASTX to map the transcripts obtained of southern hake to the Atlantic cod immune system database; hit of $e \leq 1.0e-12$ were considered as a match.

Simple Sequence Repeats Identification and Validation

The identification of simple sequence repeats (SSRs) was made using the SSRIT Perl script (Temnykh et al., 2001), which was modified to detect perfect microsatellites from dimers to decamers with at least 5 repeats. Mononucleotide repeats were not considered. These settings are in agreement with the suggested by Doyle et al. (2013).

The program Primer 3.0 (Rozen and Skaletsky, 2000) was utilized for the design of primers. For microsatellite validation, we selected 14 microsatellite markers based on two criteria: (i) availability of sequence with annotation and (ii) prioritization of di or tri-nucleotide loci, which have been described as having a potential for higher mutation rates (Ellegren, 2004). Genomic DNA was extracted in accordance with Vidal et al. (2012) from muscle tissues collected from 18 wild-caught adult individuals (41°28' S-72°56' W) associated with commercial bottom fisheries activities. Each microsatellite was amplified using the methodology described in Renshaw et al. (2011). Amplified microsatellites PCR products were genotyped on a

ABI 377 Automated Sequencer and output runs were analyzed with Genotyper 2.5 (Perkin Elmer) and Genescan 3.1.2 (Applied Biosystems). Observed (Ho) and expected (He) heterozygosities and Hardy-Weinberg equilibrium (HWE) were calculated for each locus using GenAlex ver 6.5 (Peakall and Smouse, 2012).

RESULTS AND DISCUSSION

Sequence Pre-processing and *De novo* Assembly

In last years, genomics research in aquaculture and fisheries species have attracted great interest, and a number of genomic resources had been developed. In the present study we performed, for the first time, a comprehensive spleen and liver tissues transcriptome sequencing of southern hake from mixed cDNA libraries of 10 adult samples. The run generated 950,000 raw reads (Short Read Archive SRP081286). Following the processing trimming steps, the sequence sets were reduced to 729,400 reads, with a minimum length of 50 bp and with an average length of 291 bp (**Table 1**). Currently, there is no reference genome available for this species, thus we used the *de novo* assembly (Mundry et al., 2012). The pre-processed reads were assembled using CLC *de novo* assembler and MIRA3 programs, obtaining 10,379 and 7264 unigenes, respectively, which were merged and re-assembled using CAP3 program, obtaining, in turn, a total of 10,314 unigenes with an average length of 510 bp and N50 of 572 bp (**Table 2**). The higher N50 obtained after CAP3 confirms that merging different assemblies can produce higher N50 values (Kumar and Blaxter, 2010). Although N50 is not an exact parameter to measure the accuracy of contigs (Salzberg et al., 2012), it is one of the most popular reference-free measures (Li et al., 2014). The N50 value was in the range reported for other transcriptome *de novo* fish studies (Milano et al., 2011; Fan et al., 2014; Schunter et al., 2014).

TABLE 1 | Summary of raw and pre-processed reads.

Raw reads	950,000
Duplicate reads	228,000
Reads smaller than 50 bp	190,000
Reads after pre-processing	703,000
Minimum length	50
Maximal length	725
Average length	291

TABLE 2 | Summary of the assemblies done with CLC, MIRA3, and CAP3.

	CLC	MIRA3	CAP3
Contigs	10,379	7264	10,314
Min length	50	50	50
Max length	3832	5166	5167
Average length	419	512	510
N50	502	553	572

Sequence Annotation and Transcriptome Completeness

To understand the role of the assembled southern hake unigenes, they were annotated using several public databases. All the sequences were aligned against the NCBI non-redundant protein (nr) and Swiss-Prot databases using BLASTX program, 3171 out of the 10,314 unigenes (30.74%) having significant BLAST matches (**Supplementary Table S1**). Of these, approximately 75% (2401) of the unigenes had significant BLAST matches with fish species, such as Nile tilapia, zebrafish, Atlantic salmon and spotted green pufferfish, among others (**Table 3**). The remaining 7143 (69.26%) unigenes resulted in non-significant hits, a result similar to other studies of non-model fish species (Shin et al., 2012; Qian et al., 2014). Normally this happens either due to incomplete gene information on non-model species in public database or because sequences of non-coding RNAs among the unigenes are included (Hou et al., 2011). Alternatively, a length effect has been described by Ding et al. (2015), in which short reads, normally obtained in high sequencing approaches, would reduce the BLAST annotation efficiency. However, our results do not support this last suggestion, because unigenes sequences shorter and longer than 572 bp had almost the same rate of

TABLE 3 | Top-hit species distribution of BLAST results.

Species	N° hits
<i>Oreochromis niloticus</i> (Nile tilapia)	867
<i>Danio rerio</i> (zebrafish)	376
<i>Salmo salar</i> (Atlantic salmon)	345
<i>Tetraodon nigroviridis</i> (spotted green pufferfish)	217
<i>Homo sapiens</i> (human)	159
<i>Mus musculus</i> (house mouse)	101
<i>Rattus norvegicus</i> (Norway rat)	98
<i>Osmerus mordax</i> (rainbow smelt)	70
<i>Gallus gallus</i> (chicken)	68
<i>Xenopus</i> (<i>Silurana</i>) <i>tropicalis</i> (western clawed frog)	60
<i>Oncorhynchus mykiss</i> (rainbow trout)	55
<i>Bos taurus</i> (cattle)	52
<i>Dicentrarchus labrax</i> (European seabass)	49
<i>Gadus morhua</i> (Atlantic cod)	46
<i>Ictalurus punctatus</i> (channel catfish)	41
<i>Anoplopoma fimbria</i> (sablefish)	32
<i>Takifugu rubripes</i> (Fugu rubripes)	29
<i>Xenopus laevis</i> (African clawed frog)	27
<i>Sus scrofa</i> (pig)	26
<i>Epinephelus coioides</i> (orange-spotted grouper)	23
<i>Esox lucius</i> (northern pike)	18
<i>Oryzias latipes</i> (Japanese medaka)	17
<i>Macaca fascicularis</i> (crab-eating macaque)	16
<i>Pongo abelii</i> (Sumatran orangutan)	16
<i>Perca flavescens</i> (yellow perch)	15
<i>Paralichthys olivaceus</i> (Japanese flounder)	11
<i>Oplegnathus fasciatus</i> (barred knifejaw)	10
<i>Epinephelus bruneus</i> (longtooth grouper)	9
<i>Merluccius merluccius</i> (European hake)	9
<i>Sparus aurata</i> (gilthead seabream)	9

annotation (result not shown). Therefore, our results suggest a huge potential for the disclosure of new genes, species-specific genes or non-coding sequences in this hake species.

An important challenge in *de novo* transcriptome assemblies' research consists in selecting an adequate sequencing depth to cover an appropriate range of transcripts expressed in a

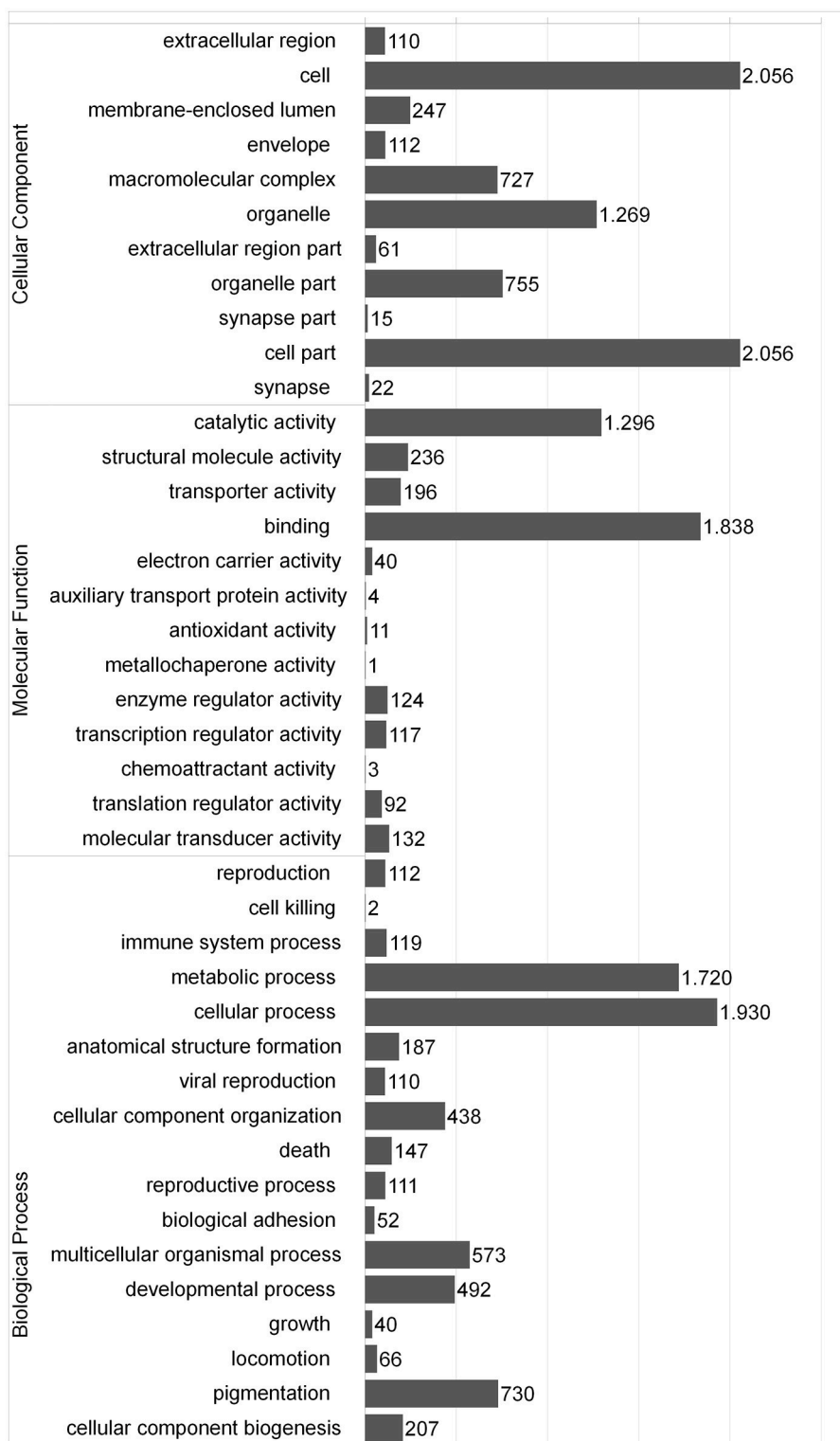


FIGURE 1 | Gene ontology classification (level 2).

particular sample and organism. There is a limited number of approaches available for evaluating the amount of reads to produce an adequate depth coverage of transcript expressed in a determined species and sample. An appropriate metric was developed by Parra et al. (2007) based on 248 CEG, of which 72% were covered by the southern hake transcripts obtained in this study. Moreover, we extracted 184 GO terms associated with immune system and 358 corresponding immune proteins for Atlantic cod. BLAST search against this immune database showed that southern hake unigenes matched 52.2% (186) of the entire Atlantic cod database. In comparison, the percentage of all the Atlantic cod genes available in public database with hits to the southern hake unigenes was noticeably less (30%). We consider that these results are due to the prevailing immune function of liver and spleen tissue in fish (Lieschke and Trede, 2009). To further characterize the assembled unigenes, the BLAST results were submitted to BLAST2GO suite for annotation of Gene Ontology (GO) terms and enzyme commission (EC) numbers. A total of 16,728 GO terms were associated with the 3171 unigenes, with the higher number of GO terms linked to Biological process (9406, 45%) followed by Cellular component (7430, 36%) and Molecular function (4090, 20%). The distribution and composition of the GO terms obtained were similar to those reported in another fish, including another hake species, European hake (Milano et al., 2011; Liao et al., 2013; Li et al., 2015). In the Biological process category, proteins involved in metabolic and cellular processes were dominant, while in the categories of Cellular component and Molecular function, cell and cell part, and catalytic activity and binding proteins, respectively, were dominant (Figure 1). Of the 3171 sequences with GO terms related, 776 had a total of 845 EC numbers. KEGG analysis linked the annotated sequences to 10,428 metabolic pathways, being a large number of sequences related to carbohydrate metabolism (205), translation (252), signal transduction (270), cancers (360), neurodegenerative diseases (262) and infectious diseases (680) (Table 4). Moreover, several transcripts (119) associated with key immune response (GO:0002376) were identified. For example, we have identified various major histocompatibility antigens transcripts, which is consistent with the great variability reported for this gene group (Cohen et al., 2006). Likewise, we have detected several transcript with crucial roles in the immune and stress response such as cytokines (tumor necrosis alpha, interleukin), complement component and heat shock proteins, among others.

Simple Sequence Repeats Identification and Validation

Generally, genetic markers, such as SSRs, have been considered useful tool for evaluating and studying genetic structure, diversity and quantitative traits loci discovery (Chauhan and Rajiv, 2010) in fishes. Moreover, SSRs developed from transcriptome sequences are meaningful for the evaluation of functional variation and represent a useful level of transferability among close species.

A total of 2302 SSRs were identified on 1687 unigenes, dimers and trimers being the most abundant class, representing together

TABLE 4 | Biochemical pathway mapping (KEGG) for southern hake unigenes.

Pathway	Mapping genes
Metabolism	956
Carbohydrate metabolism	205
Energy metabolism	138
Lipid metabolism	117
Nucleotide metabolism	81
Amino acid metabolism	163
Metabolism of other amino acids	53
Glycan biosynthesis and metabolism	51
Metabolism of cofactors and vitamins	51
Metabolism of terpenoids and polyketides	15
Biosynthesis of other secondary metabolites	14
Xenobiotics biodegradation and metabolism	68
Genetic information processing	575
Transcription	83
Translation	252
Folding, sorting, and degradation	184
Replication and repair	56
Environmental information processing	355
Membrane transport	11
Signal transduction	270
Signaling molecules and interaction	74
Cellular processes	465
Transport and catabolism	190
Cell motility	56
Cell growth and death	121
Cell communication	98
Organismal systems	973
Immune system	342
Endocrine system	137
Circulatory system	52
Digestive system	113
Excretory system	54
Nervous system	188
Sensory system	19
Development	50
Environmental adaptation	18
Human diseases	1,499
Cancers	360
Immune diseases	90
Neurodegenerative diseases	262
Substance dependence	52
Cardiovascular diseases	50
Endocrine and metabolic diseases	17
Infectious diseases	668

near 95% of the total. The most abundant dimers motifs (36%) were AC, CA, and GT, present on 304, 265, and 276 unigenes respectively, while the motifs most abundant in trimers (4%) were CCT, GAT and GGA identified on 30, 35, and 29 sequences. Among all detected SSRs, it was possible to design appropriate PCR primers for 741 (32.19%) (Supplementary Table S1). Out

TABLE 5 | Summary of the subgroup of SSRs (14) evaluated.

Locus code	Motif	Left primers (5'-3')	Right primer (5'-3')	Allele numbers	Range (bp)	Ho/He
M_A_C_000055_SSR_2	CTT	CTCGCTGCTCTTCTTGGTCT	CGCTGCGGCTAAAGCTAATG	4	204–218	0.202/0.225
M_A_C_000087_SSR_1	TGC	CGTCTCGCAGGTCACATTCT	GGACTCATTCTCCACATCCACA	3	188–217	0.195/0.340
M_A_C_000088_SSR_1	TCC	GAAGTCAGGGTTGGAGGTGG	CGGAAGGACTGAAAGAGCGT	1	117	
M_A_C_000093_SSR_1	CAG	CACAGTCAGGCAGAGGAAGG	TGGCGAGTCCTCTGTGTAGA	1	98	
M_A_C_000131_SSR_1	GT	CCTGTAACGGAGTGGACGAC	CTCTGATGTTGTCGTGCACG	6	121–202	0.572/0.547
M_A_C_000145_SSR_1	AG	TACCTCTGGTCGACCCGAAT	CTCATGGCGAGTCTAAGGG	4	113–187	0.830/0.580
M_A_C_000150_SSR_1	TAT	CACCACACCTGGACTCAGAC	CGTCTCATCGCCGCTTCTT	3	128–159	0.065/0.068
M_A_C_000159_SSR_1	CAG	CGACCACCATCAGCTACCTG	CCGCCTTGATCTTCTCCTGG	1	113	
M_A_C_000171_SSR_1	GT	ACATGACCAAGATGTTCTTTGTACA	GAGGTTGGGTGGAACAGGAG	3	152–165	0.200*/0.241
M_A_C_000172_SSR_1	GT	AACCTCCACCCTTATGCAGC	ACAAACACATACGCGCACAC	4	182–197	0.793/0.637
M_A_C_000220_SSR_1	CA	CTCACTCTTAGCTGCCGCTT	GGATCCTGCGAGTGGAGAAG	Without PCR products		
M_A_C_000250_SSR_1	TTC	CTCTCGCTGCCTCTCGTAAG	ATAGAGTGGGAGCTGGCAGA	4	119–127	0.485/0.479
M_A_C_000329_SSR_2	GA	TATCAGAGTGATCCCGCGCA	TGAACAGGACATGGTTGCGT	2	168–172	0.345/0.432
M_A_C_000431_SSR_1	GT	AGTGTGTGGTATGAGTGTGTGT	GGACAGCTTCCTTCTTCACCT	1	102	

M_A_C_: *Merluccius australis* contig.; *: deviation from HWE.

of the 14 SSRs selected for validation, 4 (28.56%) had very low polymorphism (a single allele across samples) and 1 (7.14%) did not amplify (Table 5).

In conclusion, we have conducted a transcriptome sequencing study of spleen and liver tissues in southern hake and identified 3171 genes, of which 186 were predicted to be related to immune pathways. Moreover, we also identified 741 microsatellites and evaluated a subgroup of them. Altogether, these results provide an excellent ground for the development of platforms to investigate important genetics and ecological aspects of southern hake populations.

AUTHOR CONTRIBUTIONS

RV: Participated in the conception and design of the study and drafted the manuscript; JG: Participated in the conception,

design, and coordination of the study and helped write the manuscript; DR and RG: Performed the bench work, data analysis and drafted the manuscript.

FUNDING

This work was supported by CORFO-INNOVA Chile 12IDL2-16192.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2016.00216/full#supplementary-material>

Supplementary Table S1 | This is a xlsx archive that contains additional results from microsatellite PCR primers research and gene annotation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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